

# Glucose-stimulated Insulin Secretion Correlates with Changes in Mitochondrial and Cytosolic Ca<sup>2+</sup> in Aequorin-expressing INS-1 Cells

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## Abstract

Nutrient-stimulated insulin secretion is dependent upon the generation of metabolic coupling factors in the mitochondria of the pancreatic B cell. To investigate the role of Ca<sup>2+</sup> in mitochondrial function, insulin secretion from INS-1 cells stably expressing the Ca<sup>2+</sup>-sensitive photoprotein aequorin in the appropriate compartments was correlated with changes in cytosolic calcium ([Ca<sup>2+</sup>]<sub>c</sub>) and mitochondrial calcium ([Ca<sup>2+</sup>]<sub>m</sub>). Glucose and KCl, which depolarize the cell membrane, as well as the Ca<sup>2+</sup>-mobilizing agonist, carbachol (CCh), cause substantial increases in [Ca<sup>2+</sup>]<sub>m</sub> which are associated with smaller rises in [Ca<sup>2+</sup>]<sub>c</sub>. The L-type Ca<sup>2+</sup>-channel blocker, SR7037, abolished the effects of glucose and KCl while attenuating the CCh response. Glucose-induced increases in [Ca<sup>2+</sup>]<sub>m</sub>, [Ca<sup>2+</sup>]<sub>c</sub>, and insulin secretion all demonstrate a pronounced initial peak followed by a sustained plateau. All three parameters are increased synergistically when glucose and CCh are combined. Finally, [Ca<sup>2+</sup>]<sub>m</sub>, [Ca<sup>2+</sup>]<sub>c</sub>, and insulin secretion also display desensitization phenomena following repeated additions of the three stimuli. The high sensitivity of [Ca<sup>2+</sup>]<sub>m</sub> to Ca<sup>2+</sup> influx and the desensitization-resensitization effects can be explained by a model in which the mitochondria of INS-1 cells are strategically located to sense Ca<sup>2+</sup> influx through plasma membrane Ca<sup>2+</sup> channels. In conclusion, the correlation of [Ca<sup>2+</sup>]<sub>m</sub> and [Ca<sup>2+</sup>]<sub>c</sub> with insulin secretion may indicate a fundamental role for Ca<sup>2+</sup> in the adaptation of oxidative metabolism to the generation of metabolic coupling factors and the energy requirements of exocytosis. (*J. Clin. Invest.* 1996. 98:2524–2538.) Key words: mitochondrial calcium • cytosolic calcium • insulin secretion • aequorin • signal transduction

## Introduction

The B cells of the pancreatic islet store insulin in secretory granules which are released by exocytosis when the concentra-

tion of nutrient, such as glucose or amino acids, is increased in the blood. Acetylcholine and other neurotransmitters, as well as gastrointestinal hormones, are physiological stimulators of insulin secretion (1–4). Nutrients induce a series of complex changes in B cell metabolism ultimately leading to the stimulation of secretion. Glucose sensing by the B cell is achieved through expression of specific glucose transporters and key metabolic enzymes that are poised to stimulate oxidative phosphorylation efficiently (5–9). The nutrient stimuli promote the increased synthesis of metabolic coupling factors such as ATP and fatty acyl CoA derivatives (3, 10–13). Glucose causes membrane depolarization and electrical activity in the B cell via the closure of ATP-sensitive K<sup>+</sup> channels, an effect generally believed to be due to an increase in the ATP/ADP ratio (14, 15). Electrical activity, in turn, leads to stimulation of Ca<sup>2+</sup> influx through voltage sensitive Ca<sup>2+</sup> channels (16, 17) and the triggering of insulin secretion (18). The electrical activity has been correlated to oscillations in cytosolic Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>c</sub>)<sup>1</sup> (19), and oscillations in insulin secretion in both rodent (20) and human islets (21). Extracellular Ca<sup>2+</sup>-dependent oscillations in [Ca<sup>2+</sup>]<sub>c</sub> have also been reported in glucose stimulated INS-1 cells (22), a highly differentiated rat B cell line (8, 23).

Ca<sup>2+</sup> plays an essential role in the triggering of exocytosis in the B cell (1, 18, 24). Such an elevation of [Ca<sup>2+</sup>]<sub>c</sub> has also been proposed to promote increased mitochondrial ATP synthesis to balance the increased energy demands of cell activation (25). This effect is thought to be exerted by activation of several key mitochondrial dehydrogenases including the pyruvate and 2-oxoglutarate dehydrogenase complexes, NAD<sup>+</sup>-isocitrate dehydrogenase, and inorganic pyrophosphatase, all of which are highly sensitive to changes in the [Ca<sup>2+</sup>]<sub>c</sub> in the mitochondrial matrix ([Ca<sup>2+</sup>]<sub>m</sub>) in the physiological nanomolar to micromolar range (25–27). The recent description of oscillations in NAD(P)H by glucose in single rat B cells and their mimicry by evoked [Ca<sup>2+</sup>]<sub>c</sub> spikes supports a role for Ca<sup>2+</sup>-mediated stimulation of mitochondrial dehydrogenases during nutrient stimulation of insulin secretion (28). To substantiate this hypothesis, it becomes crucial to measure [Ca<sup>2+</sup>]<sub>m</sub> in nutrient stimulated cells. However, because traditional Ca<sup>2+</sup> indicators such as fura-2 are trapped primarily within the cytosolic compartment of living cells, little is known about [Ca<sup>2+</sup>]<sub>m</sub> in B cells in response to glucose. Using recombinant aequorin targeted to the mitochondria by inframe fusion of the gene with the signal peptide of cytochrome *c* oxidase subunit VIII developed by Rizzuto et al. (29), Rutter et al. (22) showed evidence to suggest that addition of extracellular ATP could increase

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1. *Abbreviations used in this paper:* [Ca<sup>2+</sup>]<sub>c</sub>, cytosolic calcium concentration; [Ca<sup>2+</sup>]<sub>m</sub>, mitochondrial calcium concentration; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; Cch, carbachol; mtAeq, mitochondrial aequorin.

$[Ca^{2+}]_m$  to around 4  $\mu$ M in INS-1 cell populations transiently transfected with targeted aequorin. In this paper we describe data obtained with INS-1 cells stably expressing high levels of mitochondrially targeted aequorin. This cell line has allowed us to study both nutrient- and receptor agonist-stimulated alterations in  $[Ca^{2+}]_m$  directly and to compare and contrast this with data concerning  $[Ca^{2+}]_c$  obtained both from cells loaded with fura-2 and in INS-1 cells stably transfected with untargeted aequorin, which is predominantly expressed in the cytosolic compartment. The changes in  $[Ca^{2+}]_m$  and in  $[Ca^{2+}]_c$  were also compared to the dynamics of insulin secretion.

## Methods

**Cell culture.** INS-1 cells were cultured in RPMI 1640 supplemented with 10% FCS and other additions as previously described (22, 23). For stable transfection procedures,  $4 \times 10^6$  cells were resuspended in 800  $\mu$ l HEB (20 mM Hepes, pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM  $Na_2HPO_4$ , 250 mM sucrose) and incubated with 10  $\mu$ g of the appropriate plasmid, 2  $\mu$ g pSVneo for selection purposes and 70  $\mu$ l Transfectam (Promega Corp., Madison, WI) in a final volume of 10 ml DMEM. Media were replenished with 10 ml complete RPMI described above following an 8–10-h transfection period. Selection of stable, neomycin-resistant clones was initiated after 2–3 d growth by the addition of 250  $\mu$ g/ml G418. Due to the slow growth patterns characteristic of INS-1 cells (23), selection was allowed to proceed for 3–4 wk. G418-resistant clones were subsequently picked and transferred to 24-well culture dishes and once confluent transferred to 75  $cm^2$  culture flasks.

The plasmids used throughout this study were as described previously (29, 30) except that the mitochondrially targeted aequorin was also inserted into pcDNA1 rather than the pMT2 expression vector used previously (22, 29) as this significantly increased the levels of expression.

For analysis of aequorin expression, cells were plated on coverslips as described by Rutter et al. (22). Briefly, INS-1 cells were seeded at a density of  $4 \times 10^5$  cells/ml on glass or plastic coverslips and maintained for 2–4 d at 37°C in RPMI 1640 plus additions described above. Before experimentation, cells were incubated for 2–4 h in glucose- and glutamine-free RPMI 1640 containing 10 mM Hepes and 2.5 mM coelenterazine, the hydrophobic prosthetic group required for aequorin activation (31). The highest expressers of aequorin were termed EK-3 for the mitochondrial aequorin cell line and C-29 for the cytosolic aequorin cell line and these two cell lines were subsequently used in the experiments presented here.

**Luminescence measurements.** Luminescence was measured using a photomultiplier apparatus (model EMI 9789; Thorn EMI Electron Tubes Ltd., Middlesex, UK) maintained at room temperature. Cells, set on coverslips as described above, were placed in a hermetically sealed, thermostatted chamber at 37°C following the reconstitution period in the presence of coelenterazine. This was placed  $\sim$  5 mm from the photomultiplier. Cells were perfused constantly throughout the experiment at a rate of 1 ml/min in Krebs-Ringer buffer (135 mM NaCl, 3.6 mM KCl, 10 mM Hepes, pH 7.4, 2 mM  $NaHCO_3$ , 0.5 mM  $NaH_2PO_4$ , 0.5 mM  $MgSO_4$ , 1.5 mM  $CaCl_2$ , and 2.8 mM glucose). Data were collected every second using a photon counting board (EMI C660) and calibrated according to Cobbold and Rink (32) as described previously (22).

**Immunofluorescence.** INS-1 EK-3 cells plated at a density of  $50 \times 10^3$ /ml on poly-ornithine coated glass coverslips were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. After washes in PBS, cells were permeabilized in 0.1% Triton X-100 in PBS for 5–10 min. To the permeabilized cells, 0.1% BSA in PBS was added to block nonspecific binding for 10 min at room temperature. Cells were subsequently incubated with 12CA5 antibody (kindly donated by Dr. J. Pouyssegur, University of Nice, France) which recognizes the hemagglutinin tag (33) and/or anticytochrome *c* oxidase an-

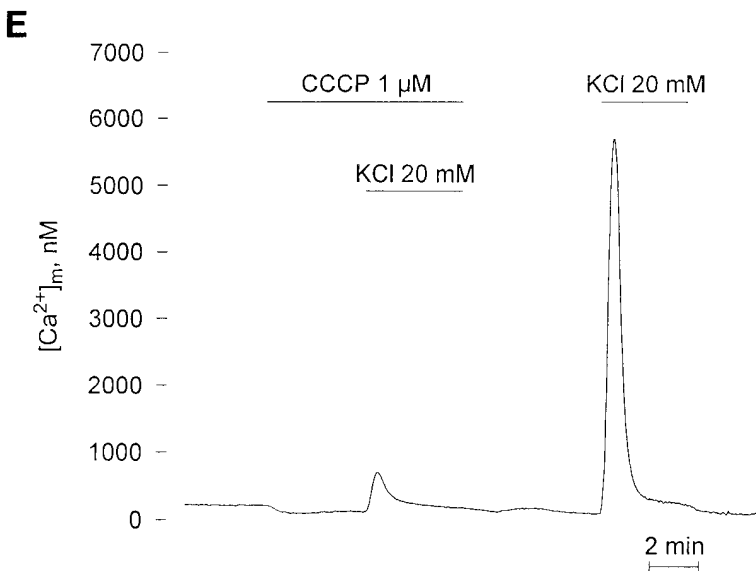
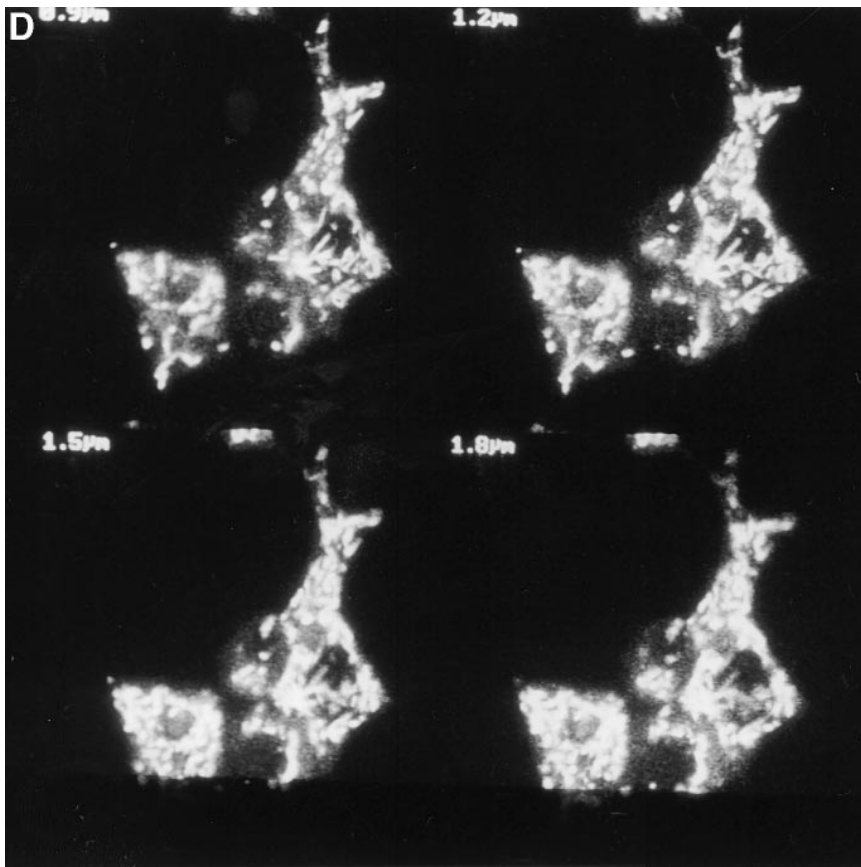
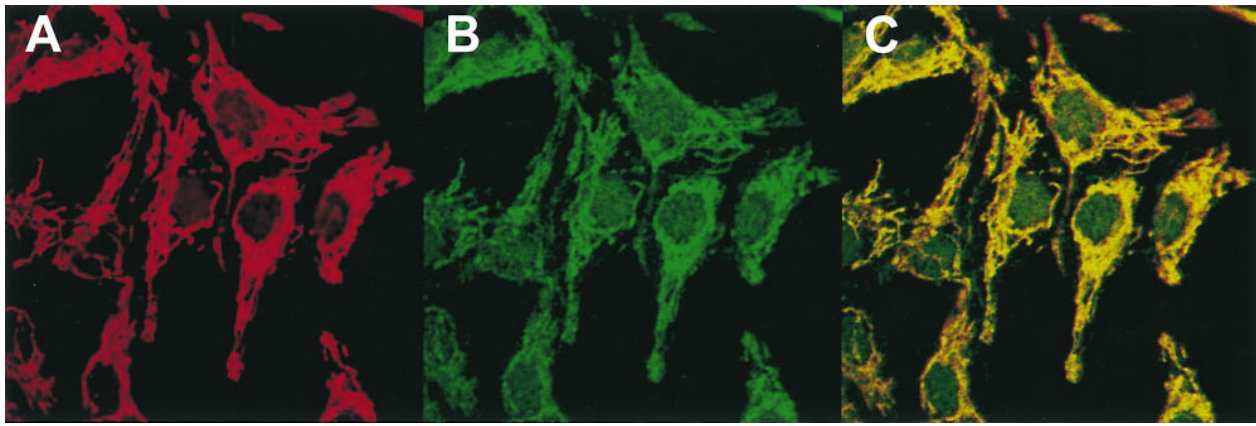
tibody (kindly donated by Dr. R. Bison, University of Padova, Italy), used at 1:50 and 1:100 dilutions respectively, for 2–3 h at room temperature. After further washing in PBS, cells were incubated with goat anti-mouse and/or goat anti-rabbit antibodies (Pierce Chemical Co., Rockford, IL) at a 1:100 dilution. Again, cells were washed repeatedly in PBS and the coverslips, mounted on glass slides, viewed using a Zeiss LSM 410 laserscan confocal microscope.

**Fura-2 measurements.** INS-1 EK-3 cells were set at a density of  $50 \times 10^3$ /ml on polyornithine coated glass coverslips in 2 ml RPMI 1640 plus additions and allowed to settle for 2–3 d. The media were then changed to RPMI 1640 glucose- and glutamine-free media as discussed above for the aequorin luminescence measurements for 2–4 h. Cells were loaded with fura-2-acetoxymethyl ester (2  $\mu$ M) for 30 min before experimentation. Fura-2 measurements by microfluorimetry were carried out according to Rutter et al. (22) and Pralong et al. (28), and fura-2 imaging experiments were carried out according to Theler et al. (17).

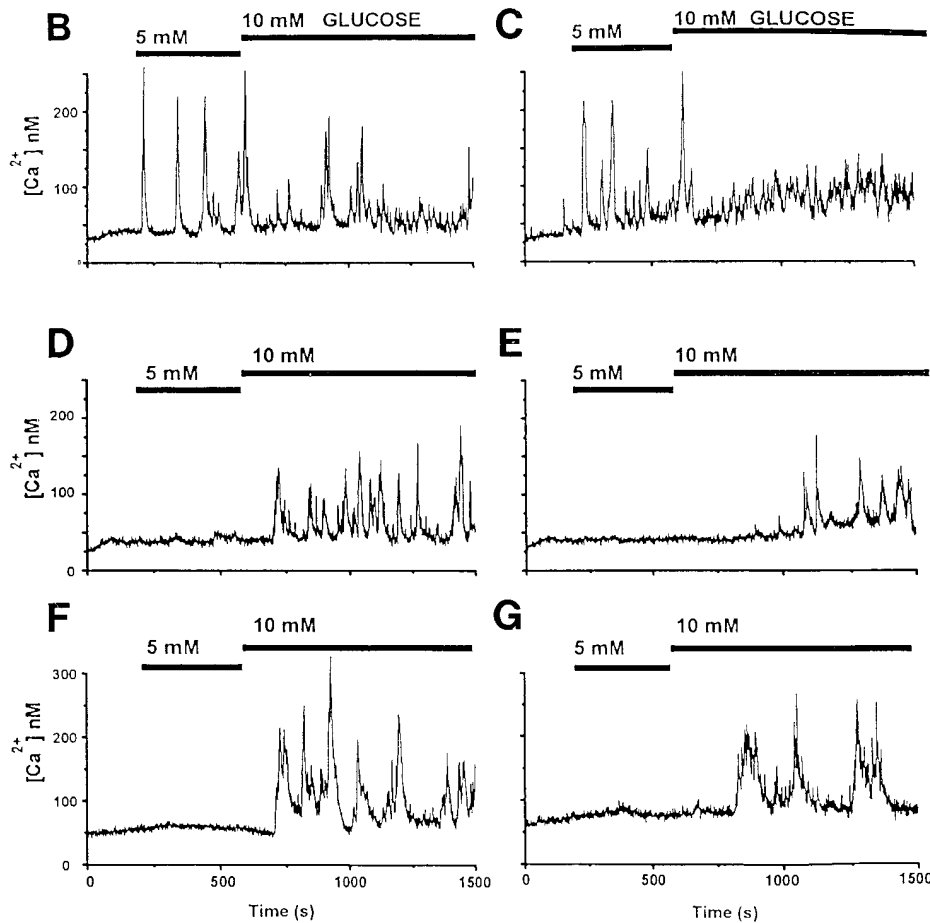
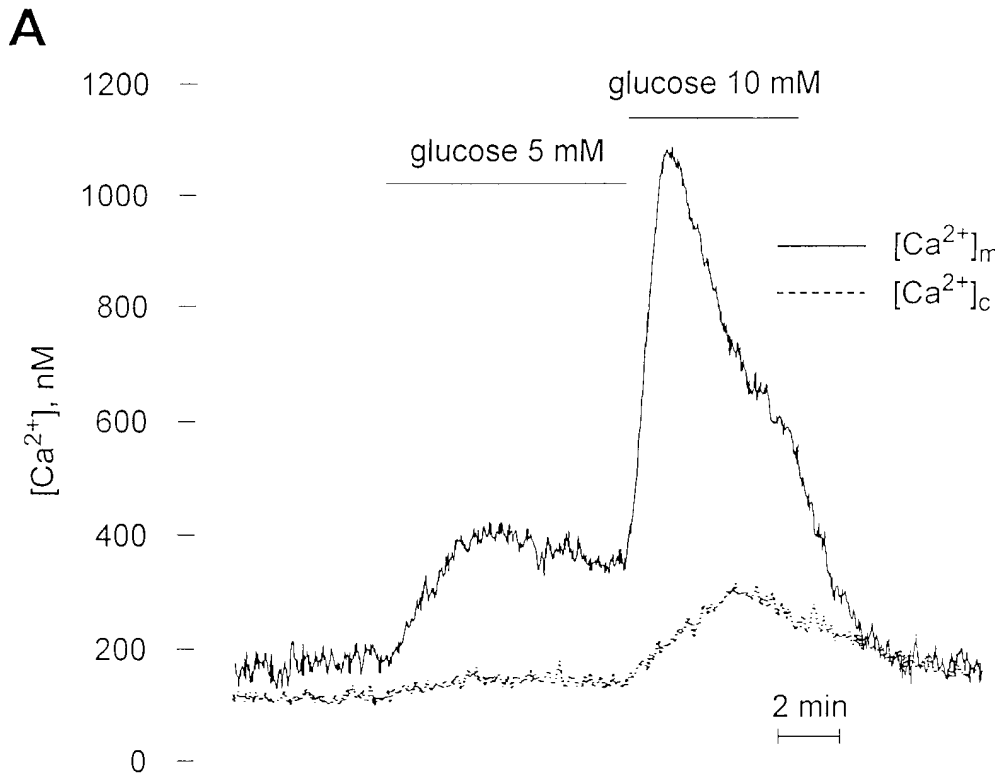
**Insulin secretion measurements.** INS-1 EK-3 and C-29 cells were used at  $1 \times 10^6$  cells/chamber and were perfused with a flow rate of 1 ml/min at 37°C as described previously. The same Krebs-Ringer buffer as for  $[Ca^{2+}]_m$  measurements was employed. Rat insulin in buffers and in acid-ethanol extracts of cells was measured by radioimmunoassay using rat insulin as standard (23).

## Results

In order to study the relationship between mitochondrial  $Ca^{2+}$  homeostasis and insulin secretion, the INS-1 cell line was cotransfected with two plasmids, the first encoding for mitochondrial aequorin (mtAeq), the other for neomycin resistance. After selection with 250  $\mu$ g/ml neomycin, several resistant clones stably expressing the chimeric photoprotein were isolated. Among these, the INS-1 EK-3 clone showed the highest level of mtAeq and presented a sensitivity to different secretagogues, glucose, KCl, and carbachol, similar to that of the parental line. The data described below were obtained with INS-1 EK-3, but similar results were obtained with another mtAeq clone. Several clones expressing cytosolic aequorin were also generated using the same procedure (see below). The mitochondrial localization of the recombinant mtAeq was verified by two different experimental approaches. In Fig. 1 (A–D), we use immunofluorescence techniques and confocal microscopy to illustrate the mitochondrial localization of the aequorin. In Fig. 1 A, INS-1 cells stably transfected with mitochondrially targeted aequorin were exposed to antibody directed against the influenza virus hemagglutinin epitope incorporated into the vector and a rhodamine-conjugated secondary antibody. Fig. 1 B shows the staining of mitochondria within the cells using an antibody raised against cytochrome *c* oxidase, a mitochondrial enzyme marker and a fluorescein-conjugated secondary antibody. Fig. 1 C shows the virtual colocalization of the two antibodies indicating a very close correlation of the two fluorescent stains and illustrating that there is no preferential expression of aequorin in a subset of mitochondria. The subcellular distribution of the mitochondria in live EK-3 cells was verified (Fig. 1 D) with the cationic dye JC-1 which is known to be specifically accumulated by energized mitochondria (34). The distribution of the mitochondria throughout the cells appears even, with an obvious exclusion from the nucleus. The morphological data do not allow to distinguish whether mtAeq is localized on the outer membrane, in the intermembrane space or within the matrix. Fig. 1 E presents functional evidence for the exclusive localization of the recombinant photoprotein within the mitochondrial ma-



**Figure 1.** Localization of targeted aequorin to the mitochondria in the stably transfected INS-1 EK-3 cell line. (A) The primary antibody is directed against HA, and detected on the rhodamine fluorescence channel (*red*). (B) The primary antibody is directed against cytochrome *c* oxidase and detected on the FITC fluorescence channel (*green*). Single optical sections were obtained simultaneously on a Zeiss laserscan confocal 410 microscope. (C) shows, in yellow, the regions where virtual colocalization of the two antigens occurs. (D) Localization of mitochondria in living cells with JC-1 (10  $\mu\text{g/ml}$ ). Optical sections were taken 0.3  $\mu\text{m}$  apart. (E)  $[\text{Ca}^{2+}]_m$  recording in INS-1 cells exposed to  $\text{K}^+$  in the presence and absence of 1  $\mu\text{M}$  CCCP. Trace is representative of five similar experiments.



**Figure 2.** Effects of glucose on  $[Ca^{2+}]_m$  as measured by aequorin, and  $[Ca^{2+}]_c$  as measured by both fura-2 and aequorin in INS-1 EK-3 and C-29 cell lines. (A)  $[Ca^{2+}]_m$  (—), and  $[Ca^{2+}]_c$  (- - -) in INS-1 EK-3 cells exposed to glucose at a final concentration of either 5 or 10 mM as indicated. (B)  $[Ca^{2+}]_c$  measurements from six single fura-2 loaded INS-1 EK-3 cells (panels A–F) stimulated with glucose are shown, and the average  $[Ca^{2+}]_c$  of 3 separate experiments in which small clusters of 3–10 fura-2 loaded INS-1 EK-3 cells are viewed (G). Additions of glucose are as indicated.

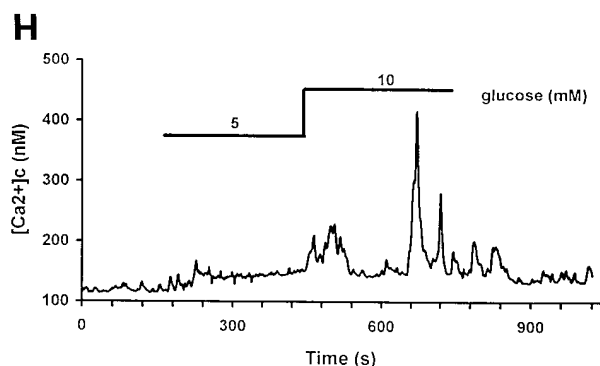


Figure 2. Continued.

trix. After reconstitution with coelenterazine, the  $[Ca^{2+}]_m$  was measured in the cell population as described in Methods. The resting  $[Ca^{2+}]_m$  measured by mtAeq corresponds to about 200 nM and decreases clearly, about 50%, upon addition of the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) which collapses the mitochondrial membrane potential (35). In the presence of the uncoupler, the  $[Ca^{2+}]_m$  rises only slightly in response to depolarizing concentrations of KCl (20 mM), while after washing away the drug, the readdition of 20 mM KCl causes a large, transient increase in  $[Ca^{2+}]_m$ , about sixfold higher than in the presence of CCCP. This pattern of  $[Ca^{2+}]_m$  is as expected for a probe localized within the mitochondrial matrix. In fact, under the same experimental conditions, a clone of cells expressing cytosolic aequorin behaved quite differently: a slight increase in  $[Ca^{2+}]_c$  is observed upon addition of CCCP, while the effects of KCl are hardly modified by the uncoupler (not shown). Taken together the data presented in Fig. 1 confirm the complete intramitochondrial localization of the recombinant mtAeq.

The most physiologically relevant stimulus for insulin secreting cells is glucose. Fig. 2 A, continuous line, illustrates the

pattern of the  $[Ca^{2+}]_m$  response to increases in the glucose concentration. Typically, 5 mM glucose will elicit a twofold average increase over the basal  $[Ca^{2+}]_m$ . Perfusing the cells with 10 mM glucose causes a further increase in the  $[Ca^{2+}]_m$  to peak levels three- to fourfold over basal. On removal of a stimulatory glucose concentration, the response returns to basal levels relatively rapidly. These data indicate that INS-1 EK-3 cells are exquisitely sensitive to perceived changes in the concentration of nutrient secretagogue responding with large reproducible changes in the  $[Ca^{2+}]_m$ .

The responses to 5 mM and to 10 mM glucose in an INS-1 clone (C-29) stably expressing high levels of untargeted, cytosolic aequorin are shown in Fig. 2 A, dashed line, for comparison. Glucose (5 mM) raises  $[Ca^{2+}]_c$  from a baseline of approximately 100 nM to a peak level of 150 nM and 10 mM glucose causes a further increase to 300 nM. In order to measure the  $[Ca^{2+}]_c$  in the clone expressing mtAeq, the fluorescent  $Ca^{2+}$  indicator, fura-2, was employed, using single cell microfluorimetry. In Fig. 2 B, six representative  $[Ca^{2+}]_c$  traces in single, fura-2 loaded INS-1 EK-3 cells can be seen. In panel G, the mean change of  $[Ca^{2+}]_c$  to 5 and 10 mM glucose in a small cluster of cells is also shown. The heterogeneity of the response is striking when measured at the single cell level and in agreement with previous studies in the parent INS-1 cell line by Rutter et al. (22). We calculated that an increase in the glucose concentration to 5 mM evoked a  $[Ca^{2+}]_c$  response in 17% of the cells. The corresponding value was 73% for cells stimulated with 10 mM glucose. Averaging the  $[Ca^{2+}]_c$ , measured with fura-2, obtained from 150 different cells, the mean peak was  $\sim 150$  nM in the presence of 5 mM glucose and 170–180 nM in the presence of 10 mM glucose. Taken together, the results presented in Fig. 2 demonstrate that, as expected, when  $[Ca^{2+}]_c$  is measured at the single cell level, the response is oscillatory and asynchronous among different cells. On the contrary, when the signal is averaged over a large number of cells, the  $[Ca^{2+}]_c$  increases are smooth and relatively small. Most likely, the  $[Ca^{2+}]_m$  rises induced by glucose are also oscillatory at the sin-

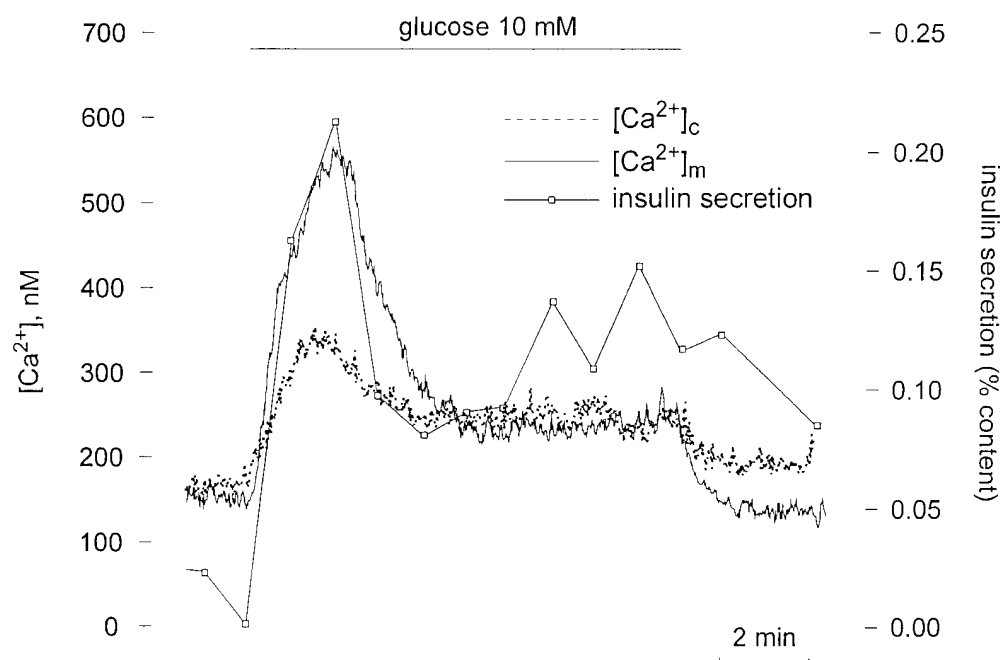
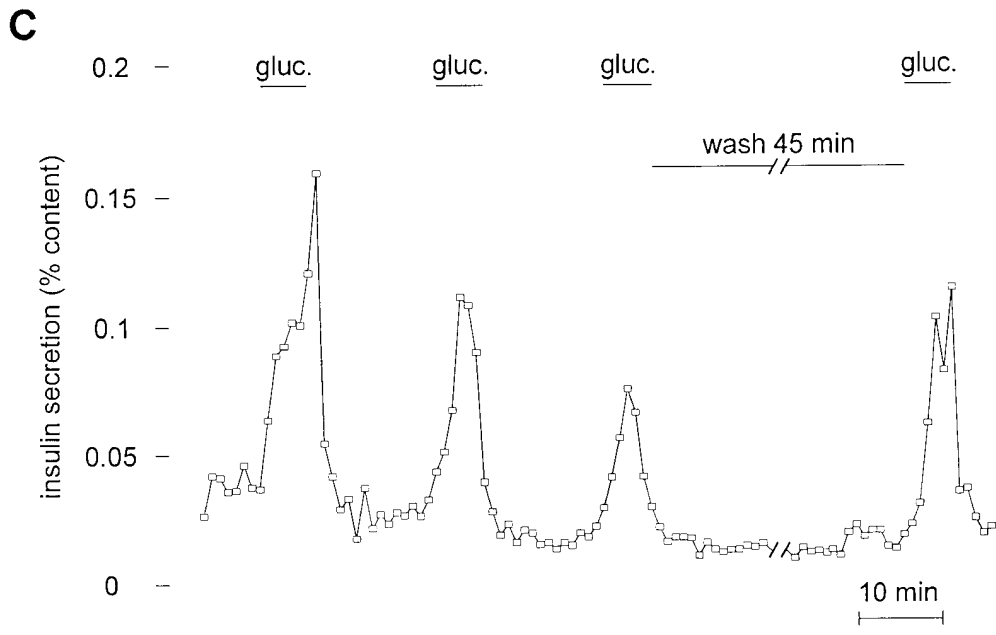
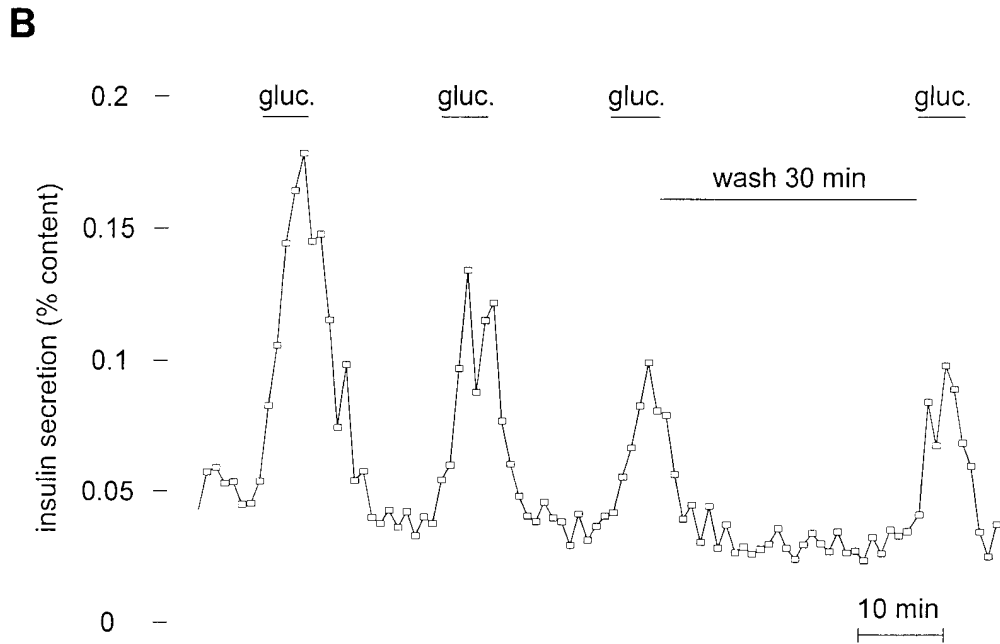
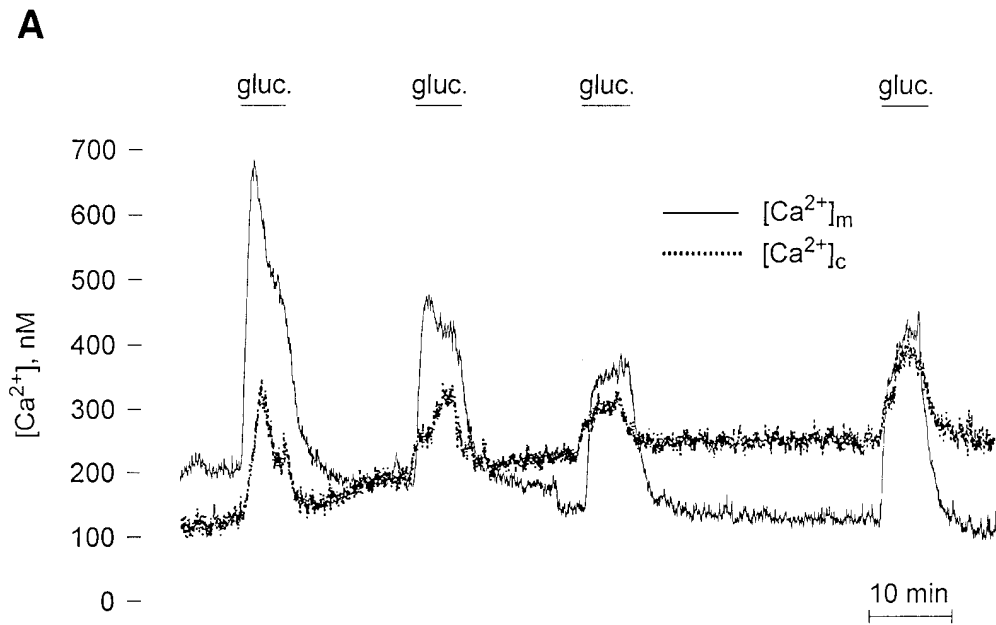


Figure 3. Effects of prolonged exposure to 10 mM glucose on  $[Ca^{2+}]_m$  (—),  $[Ca^{2+}]_c$  (---), and insulin secretion (—□) in INS-1 cells. Addition of glucose as indicated.



*Figure 4.* Effect of repeated glucose pulses on  $[Ca^{2+}]_m$ ,  $[Ca^{2+}]_c$ , and insulin secretion in INS-1 cells. (A) Representative example of the desensitization of the  $[Ca^{2+}]_m$  (—) observed in EK-3 cells and  $[Ca^{2+}]_c$  (---) observed in C-29 cells in response to three successive 5-min pulses of 10 mM glucose spaced 15 min apart during which time cells are perfused constantly in basal 2.8 mM glucose. A 30-min recovery period, again in 2.8 mM glucose, precedes the fourth exposure to 10 mM glucose. (B) The corresponding insulin secretion profile from a suspension of INS-1 EK-3 cells with a 30-min recovery period preceding the last glucose stimulus. (C) As for (B) with a 45-min recovery.

gle cell level but, at the present stage of the technique, this behavior cannot be demonstrated directly.

In Fig. 3, the kinetics of  $[Ca^{2+}]_c$ ,  $[Ca^{2+}]_m$ , and insulin secretion in response to sustained exposure to elevated glucose concentrations are shown. In this and the following experiments,  $[Ca^{2+}]_m$  and insulin secretion were measured in the EK-3 clone, while  $[Ca^{2+}]_c$  was measured in the C-29 clone. In parallel experiments, it was demonstrated that the kinetics of insulin secretion in the two clones are very similar. The pattern of  $[Ca^{2+}]_m$  increase in response to a 10-min stimulation with 10 mM glucose typically displays a biphasic response with an initial peak followed by an elevated plateau phase which remains significantly above basal  $[Ca^{2+}]_m$  until removal of the stimulus (time of onset of the response =  $26 \pm 2.8$  s,  $n = 4$ ). A similar biphasic profile is seen for  $[Ca^{2+}]_c$  (time of onset of the response =  $26 \pm 0.8$  s,  $n = 3$ ) and insulin secretion.

To delineate further the relationship between  $[Ca^{2+}]_m$ ,  $[Ca^{2+}]_c$ , and insulin secretion, we next examined the effect of successive pulses of 10 mM glucose. Fig. 4 A shows the apparent desensitization of the mitochondrial  $Ca^{2+}$  response to repeated 5-min pulses of 10 mM glucose interspersed with 15-min recovery periods. The response desensitizes by  $\sim 40\%$  between the first and second pulses and by 60% between the first and third stimulations. The response also displays a time-dependent recovery. If a 30-min recovery period is allowed following the third pulse of stimulus, the  $[Ca^{2+}]_m$ , while still desensitized, is beginning to return to a level similar to that observed with the first pulse of nutrient (first peak =  $776 \pm 63$  nM; last peak =  $563 \pm 80$  nM;  $n = 4$ ). Indeed, if the recovery period is extended to 45 min the corresponding values are  $567 \pm 44$  ( $n = 3$ ) for the first peak and  $545 \pm 19$  ( $n = 3$ ) for the last peak indicating a time dependent recovery. The desensitization profile for the  $[Ca^{2+}]_c$  is shown in the dotted line in Fig. 4 A. The relative decreases between the first and second and the first and third exposures to glucose are less marked than those observed for  $[Ca^{2+}]_m$ . Likewise, the insulin secretion profile of cells stimulated with 10 mM glucose in a similar manner demonstrates a close correlation to the  $[Ca^{2+}]_m$  except that the recovery of the insulin secretion requires a longer time frame (Fig. 4, B and C) i.e., it was incomplete even after 45 min of recovery.

In addition to the mitochondrial  $Ca^{2+}$  responses desensitizing to the nutrient stimulus, glucose, we have also found that the responses to plasma membrane depolarization with 20 mM KCl desensitize in a similar way. In Fig. 5 A, we show the effects of two successive 5-min pulses of KCl spaced 5 min apart on the  $[Ca^{2+}]_m$  in INS-1 EK-3 cells. The second pulse typically elicits only 30–35% of the effect observed during the first exposure to 20 mM KCl. If the cells are subsequently allowed a 10-min recovery period in 2.8 mM glucose, a third pulse of KCl will cause a very similar desensitized increase in the intramitochondrial  $Ca^{2+}$  levels to those observed with the second pulse.

The corresponding desensitization data for the  $[Ca^{2+}]_c$ , as measured in the clone expressing cytosolic aequorin, can be seen superimposed in Fig. 5 A, dotted line. Compared to the dramatic reduction of the  $[Ca^{2+}]_m$  response, which is reduced threefold at the second and third KCl pulses, the  $[Ca^{2+}]_c$  increase is reduced by only 30%. Depolarizing concentrations of KCl are also seen to cause a desensitization of the  $[Ca^{2+}]_c$  in fura-2 loaded INS-1 EK-3 cells (Fig. 5 B). The amplitudes of the peaks as measured with cytosolic aequorin are substantially larger than those revealed by fura-2. However, the decreases observed following successive stimulations are qualitatively

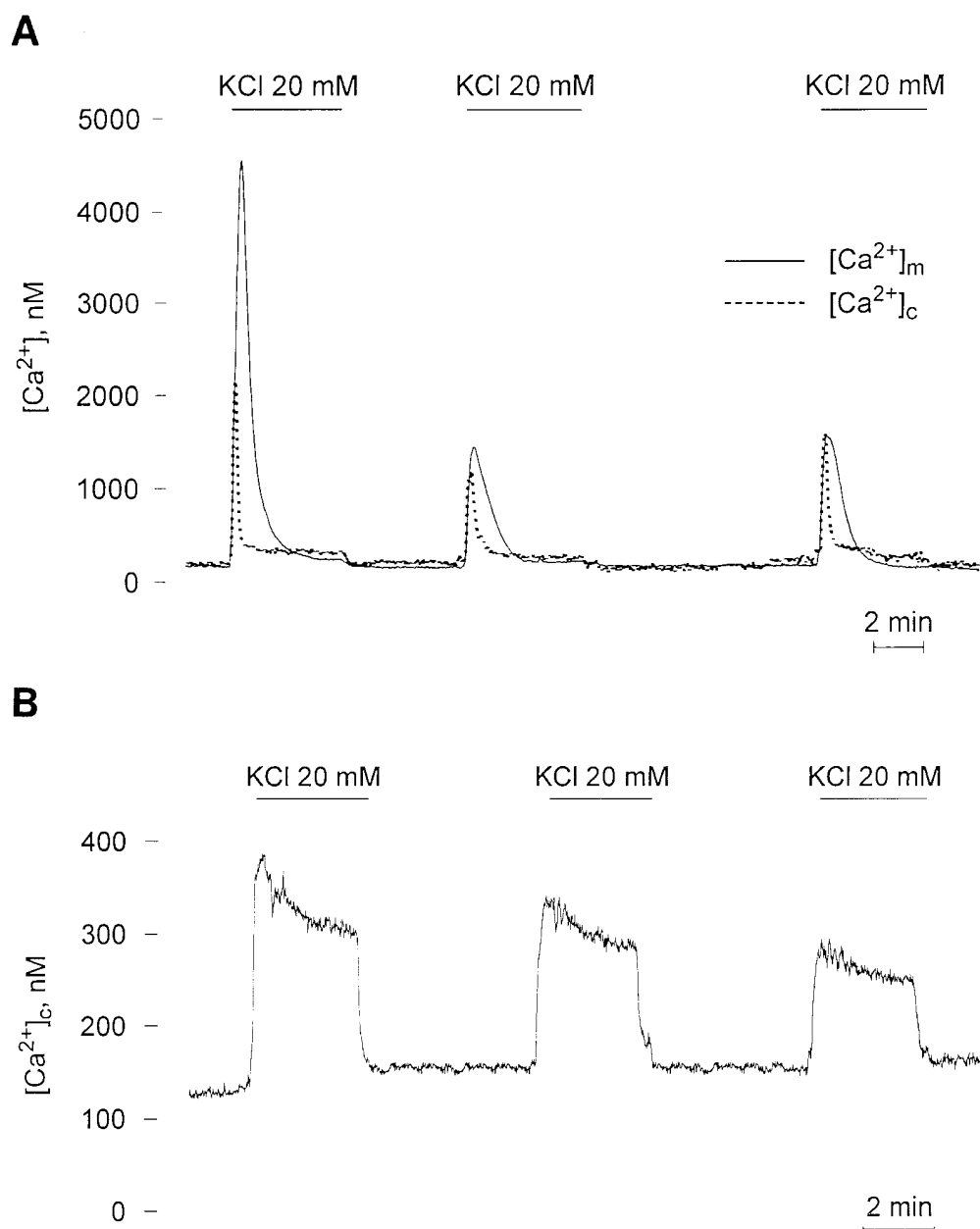
comparable. Long-term desensitization and recovery protocols such as those discussed above were not carried out in fura-2 loaded cells due to the technical difficulties of photobleaching and loss of the dye from the cells with time which complicates data analyses.

A quite different desensitization pattern to KCl is observed if 10 mM glucose is included in the perfusion medium, either in the interpulse period or throughout the experiment. Fig. 5 C in fact shows that, if only a 5-min recovery is allowed between the second and third pulses of KCl, but the cells are perfused with 10 mM glucose for 5 min before stimulation, a complete recovery of the response to 20 mM KCl is observed. This resensitization is seen for both  $[Ca^{2+}]_m$  and  $[Ca^{2+}]_c$ . In Fig. 5 D the cells are perfused in 10 mM glucose prior to the addition of the first pulse of 20 mM KCl and maintained under such conditions for the duration of the experiment. As can be seen, the presence of 10 mM glucose has a clear effect upon the magnitude of the first mitochondrial  $Ca^{2+}$  response increasing it from an average of  $5.0 \pm 0.3$   $\mu$ M ( $n = 3$ ) to  $8.6 \pm 0.2$   $\mu$ M ( $n = 3$ ). In addition, while the continued presence of glucose at high concentrations does not prevent desensitization of the response, it does, at least in part, facilitate a more rapid recovery to the original levels of  $[Ca^{2+}]_m$  resulting from the third pulse of KCl. Again, the ability of 10 mM glucose to restore the desensitized response to its original levels is apparent also for  $[Ca^{2+}]_c$  though the differences are less striking.

The relationship between  $[Ca^{2+}]_m$  and  $[Ca^{2+}]_c$  was further studied using different KCl concentrations (Fig. 6). An increase in the KCl concentration from 3.6 to 5 mM causes a doubling in  $[Ca^{2+}]_m$  without any detectable change in the  $[Ca^{2+}]_c$  as measured in INS-1 C-29 cells. Stimulation with 7.5 mM KCl causes a fourfold increase in  $[Ca^{2+}]_m$  with only a barely visible transient in  $[Ca^{2+}]_c$ . At 10 mM KCl there are marked increases both in  $[Ca^{2+}]_m$  and  $[Ca^{2+}]_c$  which are further pronounced at 12.5 mM KCl.  $Ca^{2+}$  influx induced by membrane depolarization is thus detected in a more sensitive manner by changes in  $[Ca^{2+}]_m$  than those in  $[Ca^{2+}]_c$ .

Fig. 7 shows that the activator of phospholipase C, carbachol (CCh) (100  $\mu$ M), added to cells perfused with nonstimulatory glucose concentrations (i.e., 2.8 mM) elicits transient increases in both the  $[Ca^{2+}]_m$  (Fig. 7 A) and  $[Ca^{2+}]_c$  (Fig. 7 B), as well as a transient increase in the insulin secretion. When cells are exposed to 10 mM glucose before the addition of 100  $\mu$ M CCh, not only are the  $[Ca^{2+}]_m$  and  $[Ca^{2+}]_c$  elevated in a synergistic manner, but the corresponding insulin secretion is also increased two- to threefold over that evoked by glucose or CCh alone thus demonstrating that glucose also has a potent effect upon the  $[Ca^{2+}]_m$ ,  $[Ca^{2+}]_c$ , and the insulin secretion profile in response to CCh. In addition, it was found that  $[Ca^{2+}]_m$  and  $[Ca^{2+}]_c$  rises exhibit similar desensitization when using CCh, rather than KCl, as the stimulus (data not shown).

Finally, in Fig. 8, we show evidence to suggest that the responses in mitochondrial  $[Ca^{2+}]$  to 10 mM glucose, 100  $\mu$ M CCh, and 20 mM KCl are highly dependent upon the influx of  $Ca^{2+}$  through L-type  $Ca^{2+}$  channels, known to be expressed in INS-1 cells (23). The aim of the experiments illustrated in Fig. 8 was to study the involvement of such channels using the  $Ca^{2+}$  channel inhibitor, SR7037 (36). Fig. 8 A and B shows that the responses to 10 mM glucose and 20 mM KCl are virtually abolished by the drug. The  $[Ca^{2+}]_m$  rise due to 100  $\mu$ M CCh is, as expected, only partially reduced by the presence of a  $Ca^{2+}$ -channel inhibitor since the release of  $Ca^{2+}$  from intracellular



*Figure 5.* Aequorin luminescence measurements of INS-1, EK-3 and C-29 cells stimulated with 20 mM KCl. The first two exposures last for 5 min with a 5-min recovery period between the two. The third pulse follows a 10-min recovery in 2.8 mM glucose (A), or a 5-min recovery in 2.8 mM glucose followed by preexposure of the cells to 10 mM glucose for a further 5 min (C). (D) is carried out in the continued presence of 10 mM glucose. (B) is the data obtained from one of several experiments in fura-2 loaded INS-1 EK-3 cells, where cells set and loaded as described in Methods were exposed to three consecutive pulses of 20 mM KCl. Solid lines represent data concerning [Ca<sup>2+</sup>]<sub>m</sub>, dotted lines the corresponding [Ca<sup>2+</sup>]<sub>c</sub> observed using identical protocols.

stores will be largely unaffected. However, there is an average inhibition of 50–60% of the response to CCh in the presence of 1  $\mu$ M SR7037.

## Discussion

By using the newly developed technology of targeted aequorin to monitor changes in the [Ca<sup>2+</sup>] in different subcellular compartments, we have shown that, in a variety of cell types, the [Ca<sup>2+</sup>]<sub>c</sub> increases elicited by receptor stimulation and/or plasma membrane channel opening are paralleled by large increases of [Ca<sup>2+</sup>]<sub>m</sub> (37, 38). These increases are in the range of the Ca<sup>2+</sup> affinity of the Ca<sup>2+</sup>-sensitive mitochondrial dehydrogenases including those of the Krebs cycle. Indeed, the [Ca<sup>2+</sup>]<sub>m</sub> rise correlates with enzyme activation (39) and an increase in NADH levels (38, 40). In addition, mitochondria may act to

buffer local increases in [Ca<sup>2+</sup>]<sub>c</sub> to avoid desensitization of Ca<sup>2+</sup>-directed functions and other untoward effects (41). Converging evidence now suggests that changes in [Ca<sup>2+</sup>]<sub>m</sub> mirror those of [Ca<sup>2+</sup>]<sub>c</sub> at physiological levels (29, 37, 40, 42, 43). The pancreatic B cell relies heavily upon aerobic metabolism for the generation of ATP and other factors involved in stimulus secretion coupling (3, 13).

Using INS-1 cells transiently transfected with mitochondrially targeted aequorin, it has previously been shown that stimulation with KCl or extracellular ATP which induce major synchronous rises in [Ca<sup>2+</sup>]<sub>c</sub>, cause a rise in [Ca<sup>2+</sup>]<sub>m</sub>. However, using this approach, no appreciable modification in [Ca<sup>2+</sup>]<sub>m</sub> was detected with glucose, the main physiological stimulus for insulin secretion (22). This result was unexpected given that glucose is known to cause substantial increases in [Ca<sup>2+</sup>]<sub>c</sub> in INS-1 cells (22, 23). These experiments, however, involved



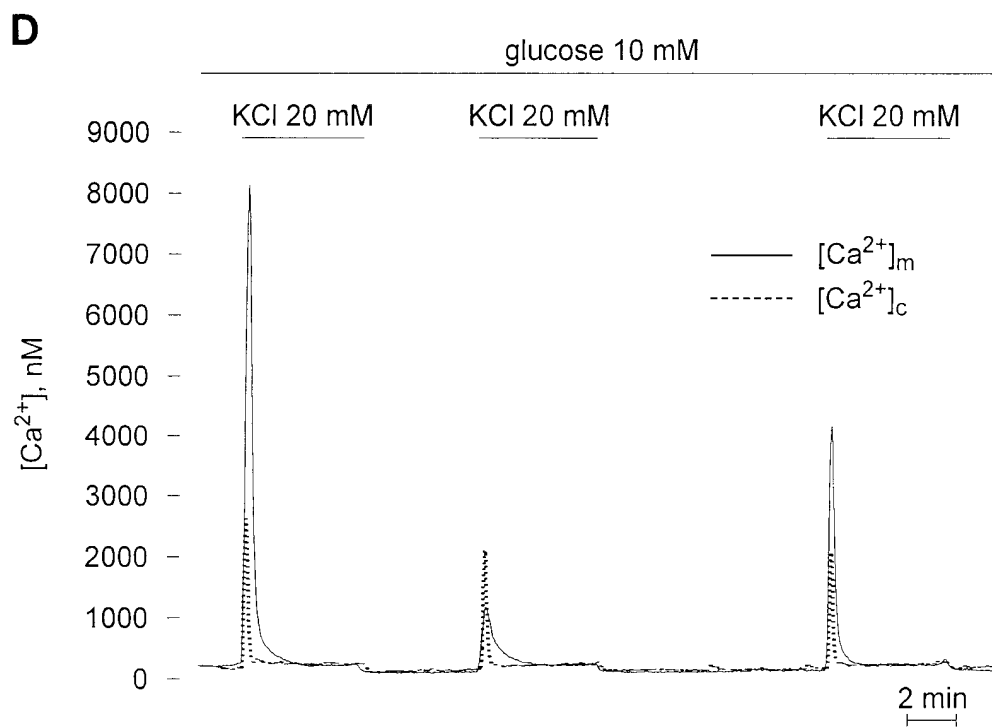
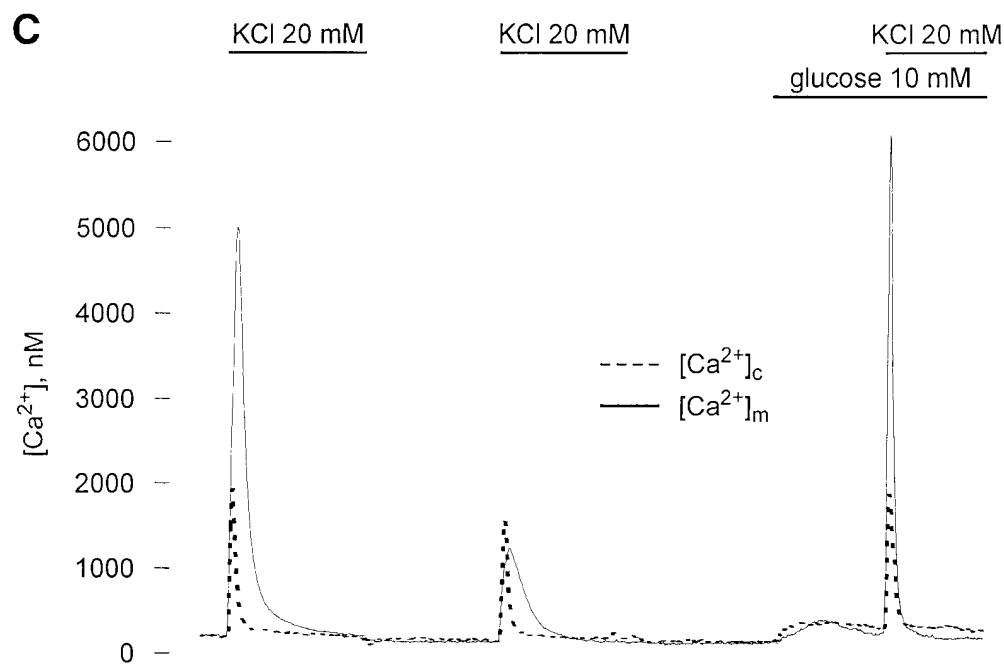


Figure 5. Continued.

transient transfection studies using a mitochondrially targeted aequorin in the pMT2 vector, under the control of the adenoviral promoter. Under these conditions the expression levels were about 10-fold lower than those obtained here using the pcDNA1 plasmid. Highly positive stable INS-1 clones expressing mitochondrially targeted aequorin were selected. In this work, clones were also isolated which express, at high levels, the untargeted cytosolic aequorin, thus facilitating comparison of mitochondrial and cytosolic Ca<sup>2+</sup> changes with the same type of probe.

By using these clones, which retained the growth patterns and insulin secretion profiles of the parental INS-1 line, we

could study directly the effect of nutrients on subcellular Ca<sup>2+</sup> handling in B cells. As previously reported, the addition of glucose in the physiological concentration range induces a dose-dependent rise in [Ca<sup>2+</sup>]<sub>c</sub>. At the single cell level, using fura-2, this increase can be shown to be due to asynchronous oscillations, which increase in frequency and amplitude at higher glucose concentrations. When the signal is averaged over a large number of cells (using either cytosolic aequorin or fura-2), oscillations are no longer detectable and the increase appears represented by small, but significant, rises above basal. Given that the [Ca<sup>2+</sup>]<sub>m</sub> could be measured, with transfected aequorin, only at the population level, oscillations in this organelle, even

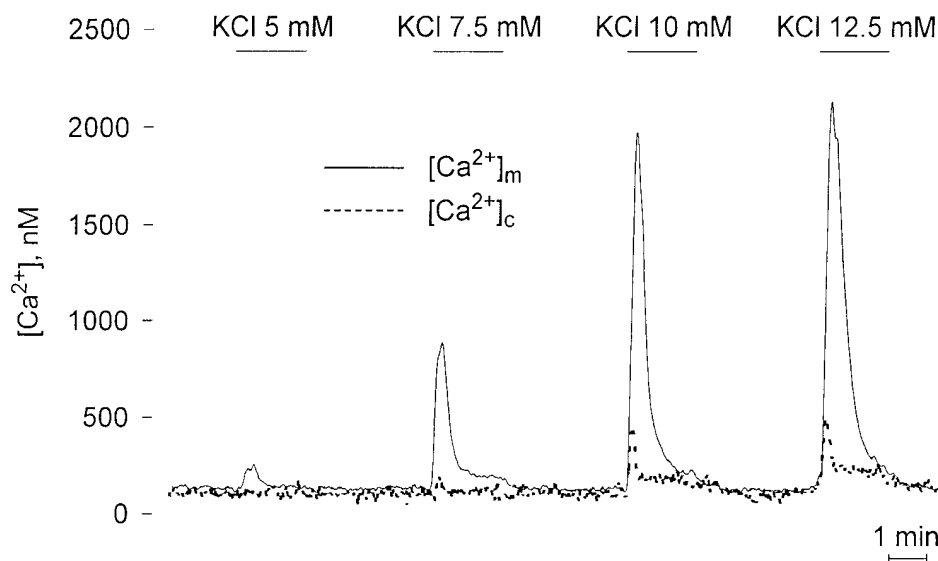


Figure 6. Concentration response characteristics of  $[Ca^{2+}]_m$  (—), and  $[Ca^{2+}]_c$  (---) in INS-1 EK-3, and C-29 cells stimulated with KCl. Traces are representative of at least four similar experiments.

if present, could not be detected. Stimulatory glucose concentrations evoke sustained increases in  $[Ca^{2+}]_m$  over the time of glucose perfusion which is in marked contrast to the rapid and transient surge in  $[Ca^{2+}]_m$  in response to KCl and CCh occurring in this and with similar agonists in other cell types (22, 29, 37, 38, 40). At 10 mM glucose, the increase was biphasic: a transient major rise (peak  $\sim 700$  nM) was followed by a sustained plateau at  $\sim 400$  nM. The mitochondrial nature of these  $[Ca^{2+}]$  changes was confirmed both by the immunocytochemical evidence of the mitochondrial localization of the recombinant probe, and by the sensitivity to the mitochondrial poison, CCCP. Moreover, the high levels of expression (which provide a good signal to noise ratio also at low  $Ca^{2+}$  concentrations) allowed to estimate  $[Ca^{2+}]_m$  to be, at rest,  $\sim 150$ – $250$  nM dependent on mitochondrial energization. Most likely, the relatively small average increases of  $[Ca^{2+}]_m$  induced by glucose are the result of a series of asynchronous, much larger spikes at the single cell level. Last, but not least, the increases in  $[Ca^{2+}]_m$  induced by high glucose are completely abolished by inhibitors of voltage gated  $Ca^{2+}$  channels, indicating that the nutrient secretagogue affects  $[Ca^{2+}]_m$  as a consequence of its effects on  $[Ca^{2+}]_c$ . The desensitization of the  $[Ca^{2+}]_m$  and insulin secretion profiles following repeated glucose applications are largely parallel as is their restoration after a recovery period. This suggests that mitochondrial metabolism may be a prime determinant for nutrient-induced insulin secretion which is known to depend upon the generation of metabolic coupling factors (3, 13). It is noteworthy that glucose affects  $Ca^{2+}$  homeostasis in two ways. In addition to causing a sustained  $[Ca^{2+}]_m$  rise, it also potentiates the effect of KCl. This mechanism could be the consequence of increased  $[Ca^{2+}]_c$  and/or a dependency upon a higher concentration of metabolites to fuel the mitochondrial respiratory chain.

The receptor agonist, carbachol, probably acting through M3 receptors, also induced both  $[Ca^{2+}]_c$  and  $[Ca^{2+}]_m$  increases. Unlike glucose and KCl, the  $[Ca^{2+}]_c$  increases caused by this agonist have a dual origin: from intracellular stores, through  $InsP_3$  production, and through plasma membrane channels. In other cell types it has been shown that  $Ca^{2+}$  mobilization from

intracellular stores is particularly effective at causing  $[Ca^{2+}]_m$  increases (37, 38). This appears not to be the case in INS-1 cells. In fact, the  $[Ca^{2+}]_m$  rises induced by carbachol are significantly inhibited by blocking plasma membrane  $Ca^{2+}$  channels and are much smaller than those induced by agents such as KCl causing exclusively  $Ca^{2+}$  influx. In turn, this finding suggests that, in INS-1 cells, the relationship between mitochondria and  $InsP_3$  gated channels is different from that of several other cell types such as HeLa, fibroblasts, *Xenopus* oocytes, and hepatocytes (37, 38, and unpublished observations). This difference, however, may be instrumental in insulin secreting cells, because agonists acting on intracellular stores act primarily as potentiators of the fuel metabolites that induce  $[Ca^{2+}]_c$  rises through opening of voltage gated  $Ca^{2+}$  channels at the plasma membrane.

The responses of  $[Ca^{2+}]_m$  to carbachol and KCl, despite their different mechanisms of action, undergo rapid desensitization upon repetitive application. Also the  $[Ca^{2+}]_c$  increases decrease in amplitude upon repetitive stimulation, but the effect on  $[Ca^{2+}]_m$  peaks is more marked. This may seem surprising, in particular in the case of KCl, which neither primarily stimulates cellular metabolism nor receptor activation. One possible explanation is that L-type  $Ca^{2+}$  channels inactivate during repeated depolarizations which has previously been demonstrated in insulin-secreting cells, albeit on a much shorter time scale (44). Consequently, diminished  $Ca^{2+}$  influx may explain the attenuation of the second insulin secretory event in response to high KCl in rat pancreatic islets (45). However, data obtained in permeabilized INS-1 cells using the mitochondrial substrate, succinate, suggest that, at least in the case of glucose, the desensitization phenomenon could occur, in part, at the level of the mitochondria (P. Maechler, E.D. Kennedy, and C.B. Wollheim, unpublished observations).

In pancreatic B cells it is well established that glucose and CCh act synergistically on insulin secretion (1). We confirm a similar synergism for  $[Ca^{2+}]_c$  in INS-1 cells and show a marked synergistic effect of CCh and glucose on  $[Ca^{2+}]_m$ . Insulin secretion in response to the two agents is also more than additive. The increased  $[Ca^{2+}]_c$  and  $[Ca^{2+}]_m$  signals could be due either

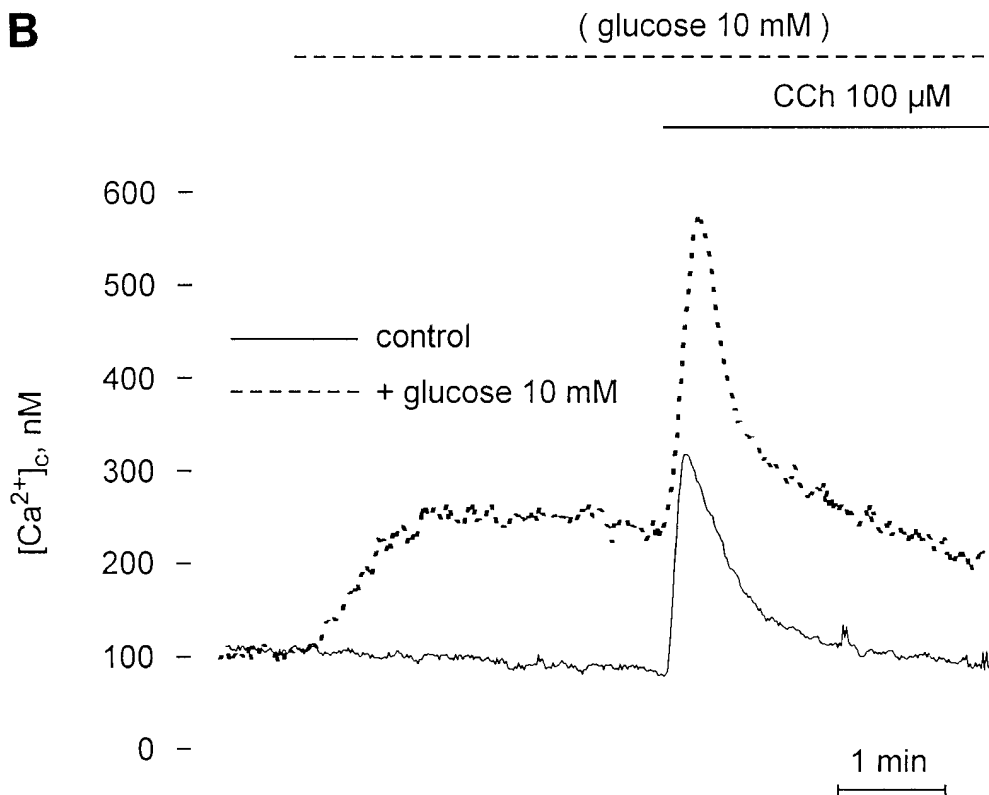
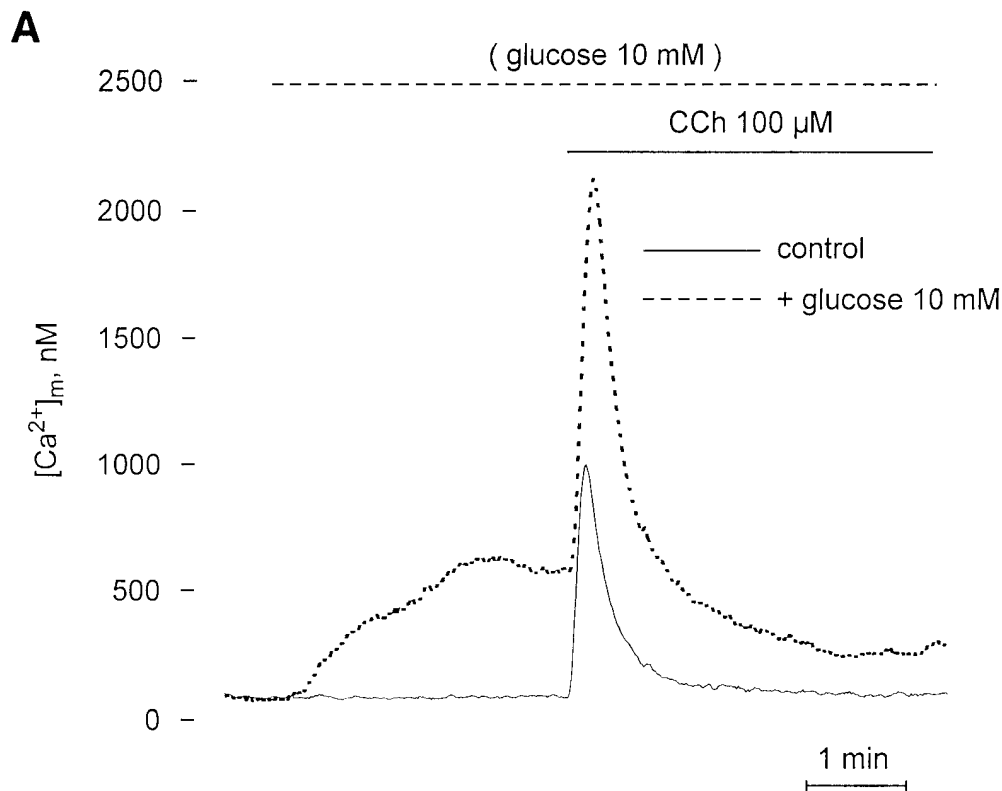


Figure 7. [Ca<sup>2+</sup>]<sub>m</sub> (A), [Ca<sup>2+</sup>]<sub>c</sub> (B), and insulin secretion profiles (C) for INS-1 EK-3 and C-29 cells stimulated with 100  $\mu$ M carbachol (CCh) in the absence (—), and presence (- - -) of 10 mM glucose at the times indicated. The data represent typical traces obtained ( $n = 3$ ).

to enhanced Ca<sup>2+</sup> influx, Ca<sup>2+</sup> mobilization or a combination of both. InsP<sub>3</sub>-producing agonists have been reported to enhance membrane depolarization evoked by nutrients (46, 47) resulting in enhanced Ca<sup>2+</sup> influx. In addition, it has been sug-

gested that CCh is more efficient at mobilizing Ca<sup>2+</sup> from intracellular stores in the presence of stimulatory glucose concentrations (17, 48). A probable mechanism for this is that glucose metabolism favors filling of the stores in the endoplas-

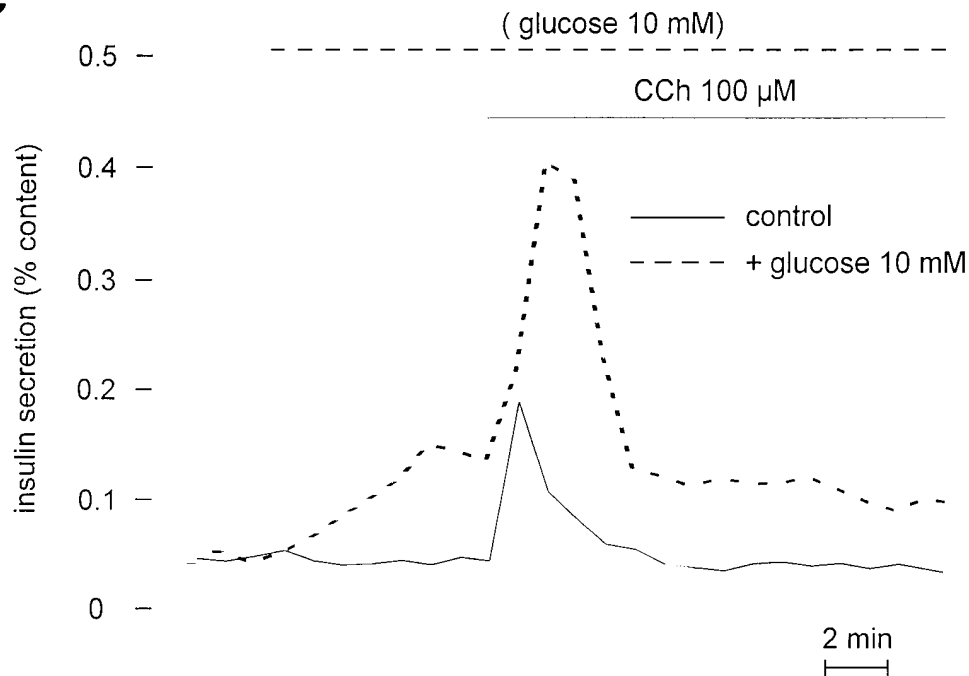
**C**

Figure 7. Continued.

mic reticulum. With the advent of aequorin successfully targeted to the endoplasmic reticulum (49), it is hoped that this hypothesis can now be tested.

The reasons for the dramatic desensitization of  $[Ca^{2+}]_m$  are not fully understood. Although it is possible that repeated exposure to agonist facilitates a temporary uncoupling of the mitochondria which protects against large, consecutive  $[Ca^{2+}]_m$  changes, this explanation appears unlikely because (a) in other cell types, repetitive  $[Ca^{2+}]_m$  spiking of similar amplitude has been observed (38), and (b) these  $[Ca^{2+}]_m$  increases are not paralleled by significant decreases of the mitochondrial membrane potential (the driving force for  $Ca^{2+}$  accumulation) (39). Another possibility is that the small  $[Ca^{2+}]_c$  desensitization, because of a mitochondrial threshold, is sufficient to account for a major effect on  $[Ca^{2+}]_m$ . This too does not appear to be the main explanation, because, if the first stimulation is reduced in intensity as to mimic the  $[Ca^{2+}]_c$  rise occurring upon the second application of the stimulus, the  $[Ca^{2+}]_m$  increase is affected to a much lower extent (Fig. 6). Another explanation would be the existence of microdomains of elevated  $[Ca^{2+}]_c$  close to the plasma membrane channels. In other words, it is possible that the  $[Ca^{2+}]_m$  increase is heterogeneous in the mitochondrial pool, and thus highly responding mitochondria, which consume a large proportion of their aequorin in the first pulse, are overlooked in the calibration of the following stimulations, as previously shown for HeLa cells (38). The resensitization of the KCl and CCh responses enhanced by glucose is probably due to glucose metabolism as the production of coupling factors may favor enhanced movement of mitochondria around the cell ensuring that other mitochondria are rapidly transported to the site where the response is initiated. Alternatively, glucose may protect the mitochondria from untoward effects of marked  $[Ca^{2+}]_m$  rises. The existence of functional

coupling between plasma membrane  $Ca^{2+}$  channels and the mitochondria is strongly suggested from the difference in the KCl concentration dependency of  $[Ca^{2+}]_m$  and  $[Ca^{2+}]_c$ . In particular, negligible increases in  $[Ca^{2+}]_c$  are paralleled by large increases in  $[Ca^{2+}]_m$ . The simplest explanation of these findings is that the increases in  $[Ca^{2+}]_c$  close to the plasma membrane evoked by small depolarizations result in undetectable changes in the  $[Ca^{2+}]_c$  but are sensed and amplified by mitochondria strategically located in those regions. Indeed, differences in the  $Ca^{2+}$  concentration between the bulk cytosol and the subplasma membrane region have been observed in fura-2 loaded insulin secreting cells using fast videoimaging (17). Similarly, exocytosis of insulin is stimulated more efficiently by  $Ca^{2+}$  influx than by  $Ca^{2+}$  mobilization in spite of the same level of  $[Ca^{2+}]$  attained (50).

As to mitochondrial distribution, the JC-1 data does not indicate preferential localization of mitochondria in any specific cell region. This, however, does not disprove the possibility that highly localized domains exist. Indeed, in HeLa cells, where mitochondrial distribution appeared a priori uniform throughout the cell, a more detailed electron microscopy study showed that a fraction of the mitochondrial pool, which well matched the proportion of highly responding mitochondria, is in close contact with the ER cisternae, the main intracellular  $Ca^{2+}$  store (37, 51). This relationship could underlie the amplified response of the  $[Ca^{2+}]_m$  relative to  $[Ca^{2+}]_c$  observed in this but not in all cell types (51). In INS-1 cells, all stimuli tested caused a much larger increase in  $[Ca^{2+}]_m$  than in  $[Ca^{2+}]_c$ . The prediction would be that the mitochondria are, to a significant extent, close to plasma membrane  $Ca^{2+}$  channels, thus strategically situated to sense the microdomains of high  $[Ca^{2+}]$  in their proximity and to fuel the exocytotic process. Future experiments involving aequorin targeted to the subplasma mem-

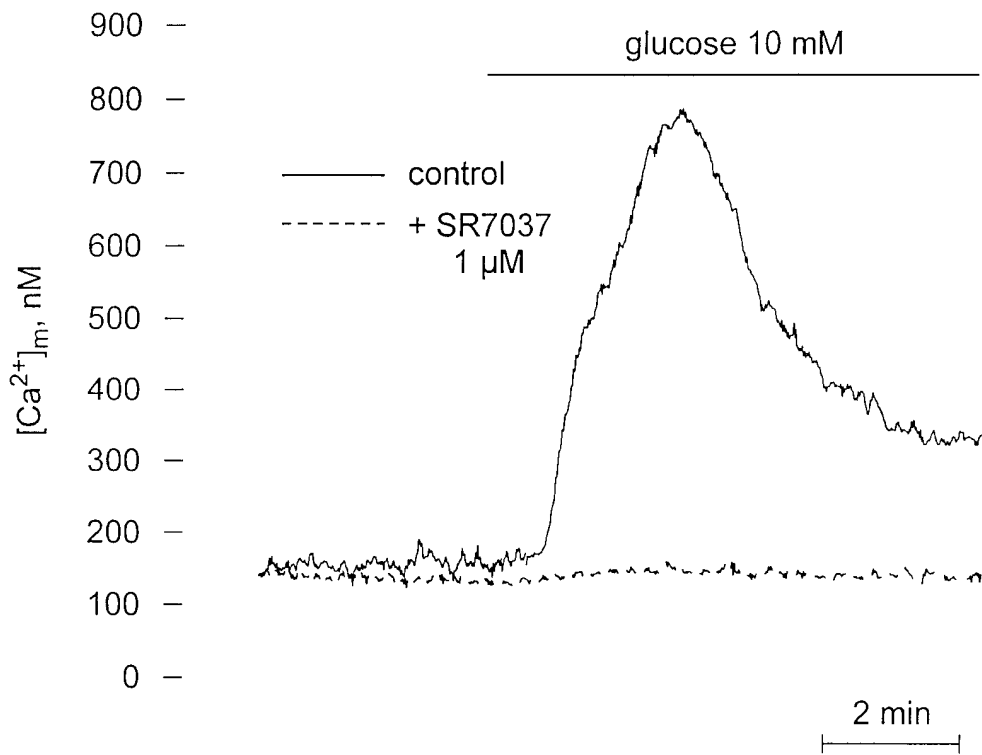
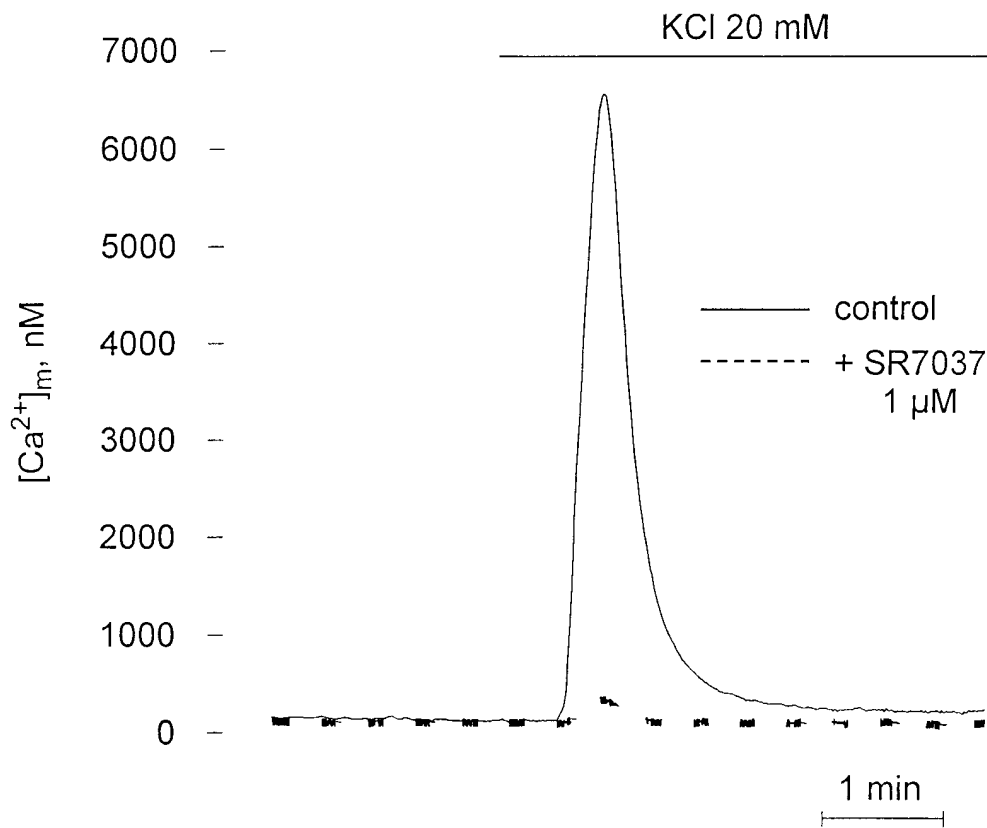
**A****B**

Figure 8.  $[Ca^{2+}]_m$  measurements in INS-1 EK-3 cells in response to 10 mM glucose (A), 20 mM KCl (B), and 100  $\mu$ M CCh (C) perfused in the presence (---) or absence (—) of SR7037 (1  $\mu$ M), an L-type  $Ca^{2+}$ -channel blocker, in the presence of 0.1% BSA. Traces are representative of at least three similar experiments.

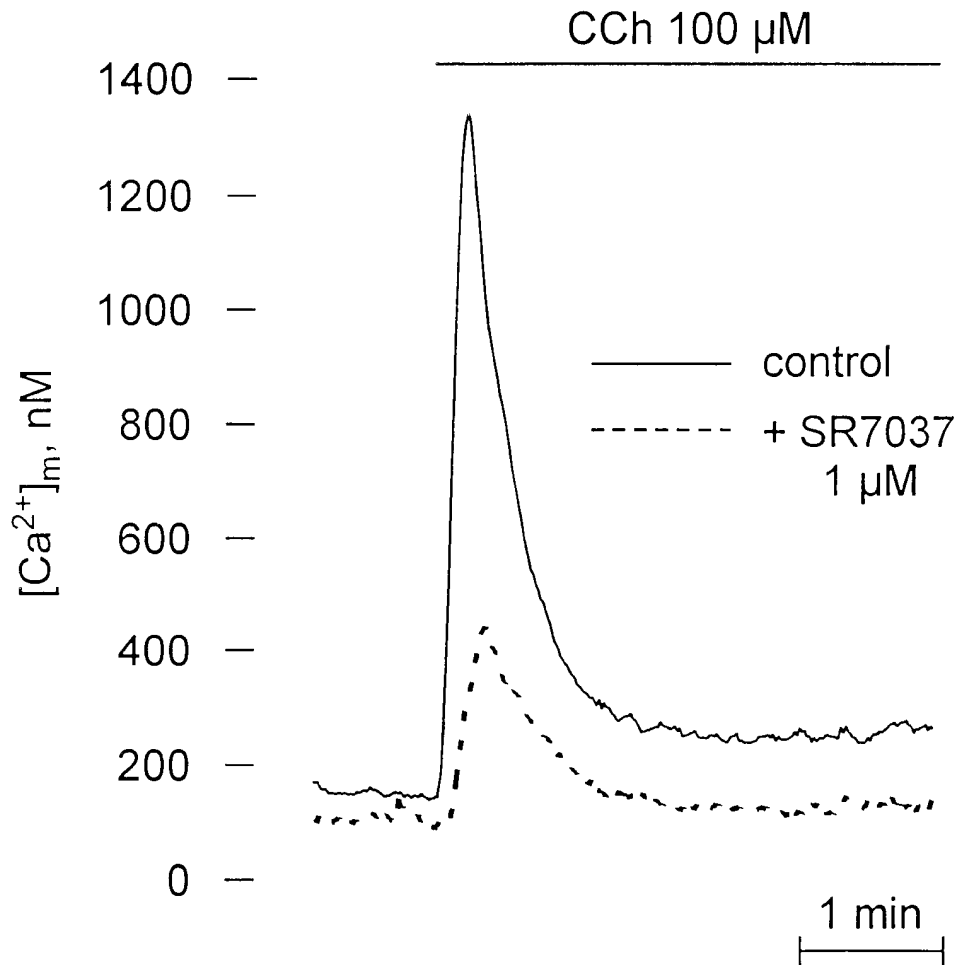
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Figure 8. Continued.

brane space should help elucidate the functional relationship between mitochondria and  $\text{Ca}^{2+}$  influx pathways in insulin secreting cells.

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